RESEARCH ARTICLE

Vangl2 cooperates with Rab11 and Myosin V to regulate apical constriction during vertebrate gastrulation

Olga Ossipova, Ilya Chuykin, Chih-Wen Chu and Sergei Y. Sokol*

ABSTRACT

Core planar cell polarity (PCP) proteins are well known to regulate polarity in Drosophila and vertebrate epithelia; however, their functions in vertebrate morphogenesis remain poorly understood. In this study, we describe a role for PCP signaling in the process of apical constriction during Xenopus gastrulation. The core PCP protein Vangl2 is detected at the apical surfaces of cells at the blastopore lip, and it functions during blastopore formation and closure. Further experiments show that Vangl2, as well as Daam1 and Rho-associated kinase (Rock), regulate apical constriction of bottle cells at the blastopore and ectopic constriction of ectoderm cells triggered by the actin-binding protein Shroom3. At the blastopore lip, Vangl2 is required for the apical accumulation of the recycling endosome marker Rab11. We also show that Rab11 and the associated motor protein Myosin V play essential roles in both endogenous and ectopic apical constriction, and might be involved in Vangl2 trafficking to the cell surface. Overexpression of Rab11 RNA was sufficient to partly restore normal blastopore formation in Vangl2-deficient embryos. These observations suggest that Vangl2 affects Rab11 to regulate apical constriction during blastopore formation.

KEY WORDS: Planar cell polarity, Vangl2, Blastopore, Xenopus, Rab11, Myosin V, Daam1, Rho-associated protein kinase

INTRODUCTION

The planar cell polarity (PCP) pathway was discovered as a molecular pathway that regulates the polarization of epithelial tissues in Drosophila embryos (Axelrod, 2009; Gubb and Garcia-Bellido, 1982; Vinson and Adler, 1987; Wang and Nathans, 2007). Cell polarization in the plane of the epithelial tissue is established by the core PCP components Frizzled, Disheveled, Van Gogh/Stbm, Prickle and Flamingo, which form separate protein complexes distributed to the opposite sides of each cell. This non-homogeneous distribution in the epithelial tissue is reinforced through positive-feedback regulation (Tree et al., 2002a). Although the roles and molecular interactions of PCP proteins in fly epithelia have been studied in some detail (Bastock et al., 2003; Jenny et al., 2005; Tree et al., 2002b; Wu and Mlodzik, 2008), accumulating evidence indicates that vertebrate PCP components have functions that are not directly related to cell polarity in the plane of the tissue. Vertebrate PCP proteins are now known to control a large number of developmental processes, including inner ear polarity (Montcouquiol et al., 2003), left-right patterning (Antic et al., 2010; Borovina et al., 2010; Hashimoto et al., 2010; Song et al., 2010), mesodermal

convergent extension (Keller, 2002; Sokol, 2000), neural tube closure (Copp and Greene, 2010; Sokol, 1996), neurite extension, neuronal migration, branching morphogenesis and vascular development (Carroll and Yu, 2012; Gray et al., 2011; Jessen et al., 2002; Ju et al., 2010; Tissir and Goffinet, 2013; Yates et al., 2010b). The molecular mechanisms underlying this pleiotropic behavior of PCP proteins in morphogenesis remain poorly understood.

Xenopus gastrulation is one of the extensively studied vertebrate models of early morphogenesis, which involves multiple cell behaviors. Gastrulation starts with the formation of the dorsal blastopore lip, characterized by the appearance of bottle-shaped cells. The bottle cells undergo apical constriction, a universal process, during which cells elongate while reducing their apical surface (Sawyer et al., 2010). At the same time, head mesoderm cells migrate towards the future anterior of the embryo. Dorsal lip converts into a ‘smile’, which subsequently spreads around the blastopore. This coordinated apical constriction promotes mesendoderm involution, which results in the entire embryo surface being covered by ectoderm. The circumference of the blastopore gradually narrows, and the blastopore is closed at the end of gastrulation, while dorsal axial and paraxial mesoderm cells undergo mediolateral interactions, known as dorsal convergent extension (Keller, 2002). Although many studies address the roles of PCP proteins in convergent extension (Gray et al., 2011; Habas et al., 2001; Sokol, 2000; Wallingford, 2012), there is sparse evidence for the function of PCP proteins in other cell behaviors during gastrulation.

The analysis of PCP signaling in vertebrate early development has been limited by insufficient knowledge of the localization of endogenous proteins at the onset of gastrulation. Therefore, we initiated an analysis of the localization and function of endogenous PCP components in morphogenetic processes that precede convergent extension in Xenopus embryos. Here, we describe an apical enrichment of Vangl2 at the blastopore lip during gastrulation and document the functional roles of Vangl2 and the downstream Wnt/PCP components Daam1 and Rock in the apical constriction of bottle cells. In embryos from which Vangl2 has been depleted, Rab11 recycling endosomes mislocalize from the apical junctions to the cytoplasm. Furthermore, we find that Rab11 vesicles function together with Myosin V to regulate apical constriction of blastopore cells. Our findings suggest that PCP signaling regulates apical constriction, at least in part, by directing Rab11-dependent trafficking to the apical surface.

RESULTS

Apical accumulation of Vangl2 at the blastopore lip

Staining of gastrula-stage embryo sections with anti-Vangl2 antibodies revealed an accumulation of immunoreactivity at the apical surface near the blastopore lip, although some signal was also detected at the basolateral cell cortex (Fig. 1A). The enrichment of Vangl2 at the apical surface was similar to the accumulation of apical pigment granules (Fig. 1B) and phosphorylated Myosin II
regulatory light chain (pMLC, Fig. 1C), which mark bottle cells undergoing apical constriction at the beginning of gastrulation (Choi and Sokol, 2009; Lee and Harland, 2007). By contrast, staining for β1-integrin showed that this protein remained basolateral in both superficial ectoderm and constricting bottle cells (Fig. 1D; supplementary material Fig. S1A,B), indicating that the observed changes are specific to Vangl2.

In embryonic ectoderm, Vangl2 was distributed to the basolateral domain of superficial cells and was enriched at the outermost ends of inner cells (Fig. 1E; supplementary material Fig. S1A). To confirm staining specificity, we generated mosaic embryos, in which some cells were depleted of Vangl2 by the use of a previously characterized antisense morpholino oligonucleotide (MO) (Darken et al., 2002). The immunostaining for Vangl2 decreased substantially in these cells, confirming that the antibody is specific to Vangl2 (Fig. 1E,E′). On western blots, this antibody detected GFP- and HA-tagged Vangl2 in gastrula-stage embryo lysates and revealed a specific 60 kDa protein band, which corresponds to endogenous Vangl2 (supplementary material Fig. S1C). The apical distribution of Vangl2 was not detected before the onset of gastrulation, at stages 9.5 and 10− (supplementary material Fig. S1D,E; data not shown). At stage 10+, the apical localization of Vangl2 was restricted to the dorsal blastopore lip, but it became circumferential at later stages, correlating with the formation of bottle cells at the blastopore ring (supplementary material Fig. S1F,G). These results indicate that Vangl2 is basolaterally localized in superficial blastula cells, but becomes apically accumulated in blastopore bottle cells.

Vangl2 is essential for both endogenous and ectopic apical constriction during gastrulation

Given the localization of Vangl2 in apically constricting cells during gastrulation, we examined the role of Vangl2 in blastopore formation and tissue involution. Interference with Vangl2 function using Vangl2 MO resulted in the inhibition of blastopore formation at the onset of gastrulation in ~80% of injected embryos (n=99, Fig. 2A,B). A knockdown of Daam1, another component of Wnt/PCP signaling (Habas et al., 2001), similarly resulted in the suppression of bottle cell formation (58%, n=50, Fig. 2C). By contrast, control MO or GFP RNA did not have significant effects on blastopore formation (Fig. 2A,D). Corresponding to these morphological changes, staining for pMLC and F-actin was suppressed in Vangl2 morphants (Fig. 2E,F; supplementary material Fig. S2F,G). Bottle cells were also locally inhibited at later stages in embryos injected with Vangl2 MO into either dorsal or ventrolateral blastomeres at the 8-16 cell stage (supplementary material Fig. S2A-E). Because the overexpression of Vangl2 did not alter the expression of mesodermal and endodermal fate markers (Darken et al., 2002), our results suggest that Vangl2 is involved in the apical constriction of bottle cells during gastrulation, rather than playing a role in cell specification, as reported in different contexts (Cortijo et al., 2012; Lake and Sokol, 2009).
To extend our observations regarding the role of Vangl2 at the onset of gastrulation, we further examined whether additional PCP components, such as Daam1 and Rock (Habas et al., 2001; Marlow et al., 2002; Winter et al., 2001), are involved in blastopore formation. Using the known dominant interfering constructs, we established a requirement for both Daam1 and Rock in apical constriction, both at the beginning and during the course of gastrulation (Fig. 3). These findings support the view that the Wnt/PCP pathway is involved in this morphogenetic process.

Bottle cells originate as a subpopulation of endodermal cells, which are influenced by many embryonic inducing and patterning factors secreted from the organizer and the surrounding tissues (Harland and Gerhart, 1997). To dissociate the fate specification process that occurs in bottle cells from morphogenetic events, we took advantage of an ectopic model, in which embryonic ectoderm undergoes apical constriction in response to overexpression of Shroom3 (here referred to as Shroom for simplicity), an actin-binding PDZ-containing protein (Haigo et al., 2003; Hildebrand and Soriano, 1999) (Fig. 4). As expected, Shroom RNA triggered efficient constriction and the accompanying hyper-pigmentation of animal pole ectoderm at the time corresponding to the beginning of gastrulation. This ectopic apical constriction was substantially inhibited by Vangl2 MO, but not by the control MO (Fig. 4A-D), reinforcing the view that Vangl2 functions in this morphogenetic process. Moreover, the amino-terminal fragment of Daam1 and a dominant interfering construct of zebrafish Prickle1, from which the PET/LIM domains have been deleted (Carreira-Barbosa et al., 2003; Takeuchi et al., 2003), also suppressed Shroom-mediated ectopic apical constriction in embryonic ectoderm (Fig. 4E-H), supporting a general role for Wnt/PCP signaling in the apical constriction of blastopore bottle cells.

**Rab11 is apically localized at the blastopore lip in a Vangl2-dependent manner**

Previous work has documented a role for endocytosis in apical constriction during gastrulation (Lee and Harland, 2010), and we wanted to assess the possibility that components of vesicular trafficking might function downstream of PCP signaling in apical constriction. We examined the localization of Rab11, a marker of the recycling endosome (Bryant et al., 2010; Mizuno-Yamasaki et al., 2012), which has been implicated in planar polarization of *Xenopus* multiciliated skin cells (Kim et al., 2012) and the neural tube.
In early ectoderm and in the non-constricting epithelial layer of the marginal zone cells, Rab11 is detected largely at cell junctions (supplementary material Fig. S3A-C) (Ossipova et al., 2014). At late blastula stages, we observed the broad apical accumulation of Rab11 at the marginal zone (supplementary material Fig. S3A-C). Rab11 became strongly enriched near the blastopore lip at the onset of gastrulation (Fig. 5A-C). Co-staining of embryo sections with anti-pMLC antibodies revealed tight colocalization with the bottle cell population (Fig. 5D-D′), indicating that Rab11 might function largely in apically constricting blastopore cells.

We next tested whether Vangl2 might influence the localization of Rab11 at the apical surface of blastopore cells. In Vangl2-depleted embryos, the apical staining for Rab11 became largely undetectable (Fig. 5E,F). Similarly, in superficial ectoderm cells, the localization of Rab11 changed from junctional to cytoplasmic after Vangl2 MO injection (Fig. 5G,H). These findings support the hypothesis that Rab11 is regulated by PCP signaling and could play a role in blastopore formation.

**Rab11 and the associated motor protein Myosin V function during blastopore lip formation and apical constriction**

Based on our observations, we hypothesize that Vangl2 functions in gastrulation by regulating the localization of Rab11. Therefore, we studied a function for Rab11 in blastopore formation using a dominant-negative mutant, Rab11S25N (Kim et al., 2012). Rab11S25N, but not the wild-type Rab11, interfered with the appearance of the dorsal lip and delayed blastopore closure (Fig. 6A-C,G). This corresponded to the disappearance of pMLC staining from the blastopore lip (Fig. 6D-F). Blastopore formation was also inhibited when Rab11 was depleted with a previously characterized specific MO (Kim et al., 2012), further supporting a role for Rab11 in apically constricted blastopore cells (Fig. 6G).

These effects were morphologically similar to the effects of a dominant-negative Dynamin construct, which interferes with clathrin-dependent endocytosis (data not shown). No change was detected in the expression of several molecular markers, including Chordin, Goosecoid, FoxD3, Xbra and Gef3.2 (GenBank accession number: NM_001096674.1), which are characteristic for mesendodermal cells abutting the blastopore (Fig. 6H). These observations indicate that Rab11 acts in morphogenetic processes rather than patterning processes leading to blastopore formation.

Given that Rab11 is required for blastopore formation and that its apical enrichment depends on Vangl2, we hypothesized that Vangl2 controls apical constriction by modulating Rab11 localization and function. We then tested whether Rab11 RNA can rescue Vangl2-deficient embryos. Whereas embryos injected with Vangl2 MO failed to form the dorsal blastopore lip at stage 10/10+, the majority of Vangl2-depleted embryos that were co-injected with either wild-type or constitutively active Q70L Rab11 RNA, but not with GFP RNA, showed normal onset of gastrulation (Fig. 6I; supplementary material Fig. S4). These findings are consistent with the hypothesis that Vangl2, at least in part, controls gastrulation and bottle cell morphology by modulating Rab11-dependent vesicular trafficking.

Besides establishing the requirement for Vangl2 in Rab11 localization, we examined whether Rab11 knockdown would affect Vangl2 subcellular distribution (supplementary material Fig. S5). The membrane-associated Vangl2 staining was consistently reduced in apically constricted bottle cells and in non-constricting ectoderm cells in which Rab11 was depleted. These findings suggest that Vangl2 is delivered to the cell surface by Rab11-mediated endocytic recycling.

We next wanted to confirm the role of Rab11 in the constricting cells using an alternative approach. Because Myosin V is a motor protein that functions together with Rab11 in apical vesicular transport, we studied a function for Rab11 in blastopore formation using an alternative approach. Because Myosin V is a motor protein that functions together with Rab11 in apical vesicular transport, we studied a function for Rab11 in blastopore formation using an alternative approach. Because Myosin V is a motor protein that functions together with Rab11 in apical vesicular transport, we studied a function for Rab11 in blastopore formation using an alternative approach.
trafficking (Bryant et al., 2010; Li et al., 2007; Roland et al., 2011), we assessed Myosin V involvement in blastopore formation and apical constriction. We expressed Myosin V tail (MyoVT), a dominant interfering construct lacking the motor domain, which mimics the loss-of-function phenotype in mouse cells (Lapierre et al., 2001; Wu et al., 1998). Blastopore formation and apical accumulation of Rab11 were inhibited at the site of MyoVT RNA injection (Fig. 7A-E). MyoVT aggregates accumulated in the cytoplasm of the injected cells and colocalized with endogenous Rab11, altering its normal apical distribution at the blastopore (Fig. 7D,E). No changes in Rab11 protein distribution were detected in samples injected with control RNA (data not shown). Finally, we observed an inhibitory effect of MyoVT on ectopic apical constriction of embryonic ectoderm in response to Shroom (Fig. 7F,G). Taken together, these experiments support the view that Rab11 cooperates with Myosin V to regulate apical constriction during gastrulation.

DISCUSSION

To extend the known roles of PCP proteins in convergent extension movements during vertebrate gastrulation, this study assessed the localization and function of the core PCP component Vangl2 in the formation of bottle cells during Xenopus gastrulation. Using antibodies specific to Xenopus Vangl2, we show that Vangl2 is basolaterally localized in superficial ectoderm but appears to become enriched at the apical surface of blastopore cells undergoing apical constriction. This change parallels the apical accumulation of F-actin and pMLC (Lee and Harland, 2007). Although β1-integrin is similarly localized at the basolateral surfaces, it is not enriched at the apical membrane during blastopore formation. Because the apical surface of bottle cells contains numerous microvilli and microfolds (Perry and Waddington, 1966; Schroeder, 1970), many apical proteins should appear to be concentrated or condensed. Therefore, it might be difficult to prove that Vangl2 accumulates in the apices of the constricted cells above the levels detected in the apices of the unconstricted cells, but this is not essential for our proposed mechanism of Vangl2 function. Of note, in deep layer ectoderm cells, Vangl2 is enriched at the lateral and the outermost surfaces, a localization that might be relevant to the potential role of Vangl2 in cell migration and intercalation. Consistent with this possibility, we observed that the ectodermal tissue depleted of Vangl2 consists of multiple cell layers by the end of gastrulation (data not shown). This differs significantly from the two-layered composition of normal ectoderm and indicates a potential defect in radial cell intercalation. Defects in epiboly (i.e. radial intercalation) have been reported previously for zebrafish embryos depleted of Celsr, another core PCP protein, but these have been attributed to Celsr function in cell adhesion rather than the PCP pathway (Carreira-Barbosa et al., 2009).

Although the significance of apical localization for Vangl2 function is currently unclear, the depletion of Vangl2 and Daam1 and interference with the function of Rock lead to the inhibition of blastopore formation and defective gastrulation. Although we observe both early and late blastopore defects in Vangl2 morphants, embryos are still able to complete gastrulation. This might be due to the incomplete depletion of Vangl2 or due to the redundant activity of Vangl1 in apical constriction at the end of gastrulation. Alternatively, a different morphogenetic process, known as convergent thickening (Keller and Danilchik, 1988), might compensate for the lack of bottle cell function during blastopore closure. This is consistent with previous work in which the microsurgical removal of bottle cells did not stop gastrulation,
suggesting that bottle cells assist tissue involution but are not required for blastopore closure (Hardin and Keller, 1988; Keller, 1981). Our additional experiments established that Prickle and Daam1 appear to be necessary for the ectopic apical constriction triggered by Shroom in embryonic ectoderm in the absence of mesoderm-inducing and patterning factors. Taken together, these observations establish a novel general function for PCP signaling in apical constriction. This role appears to be conserved in mammals, as indicated by the abnormal apical constriction in the neural tube of Vangl2 and Ptk7 mutant mouse embryos (Williams et al., 2014).

With respect to the underlying mechanism, we propose that Vangl2 is required for the apical accumulation of Rab11, which functions together with the motor protein Myosin V in blastopore formation. This model is consistent with the data of Lee and Harland, who reported the requirement for Rab5-dependent endocytosis in apical constriction (Lee and Harland, 2010). Whereas Rab5, a key component of the early endosome, was proposed to remove the excessive apical membrane that forms in constricting bottle cells (Lee and Harland, 2010), we demonstrate that Rab11, a component of the recycling endosome, is also required for apical constriction. Therefore, both the removal of membrane molecules from the surface and their recycling back to the cell membrane are essential for this process. As Vangl2 is dislodged from the cell membrane in Rab11 morphants, we propose that Vangl2 is one of the cargo molecules that is delivered to the cell surface by Rab11-mediated endocytic recycling. These results emphasize the significance of the positive-regulatory loop between PCP signaling and endocytic trafficking (Classen et al., 2005; Gray et al., 2009; Lee et al., 2003; Mahaffey et al., 2013).

Our data reveal a new role for vertebrate Vangl2 in epithelial folding, which is shared by several other PCP proteins. We show that a dominant-negative construct of Prickle, a Vangl2-associated PCP protein, suppresses Shroom-dependent apical constriction. Moreover, Rock and Daam1, two downstream mediators of PCP signaling, also function in the formation of bottle cells and appear to be essential for apical constriction. These results are consistent with reported delayed blastopore closure in embryos in which the functions of Daam1 and Dishevelled have been compromised (Habas et al., 2001; Sokol, 1996). Moreover, Wnt ligands and apical-basal polarity proteins that are known to interact with PCP molecules have been implicated in the same process (Choi and Sokol, 2009; Dollar et al., 2005). These observations further expand the spectrum of PCP-associated morphogenetic processes. Besides planar epithelial polarity, PCP proteins were reported to affect mitotic spindle orientation and cell differentiation (Bellaiche et al., 2004; Cortijo et al., 2012; Lake and Sokol, 2008), cilia positioning (Hashimoto et al., 2010; Song et al., 2010), axon guidance (Cha et al., 2009), apical-basal polarity (Tao et al., 2009), oocyte development (Shen et al., 2011) and migration of endodermal (Trichas et al., 2012), germ cell (Laird et al., 2011) and neuronal progenitors (Homada et al., 2003; Glassco et al., 2012; Jessen et al., 2002). Additionally, Vangl2 and Rock are involved in lung branching morphogenesis (Yates et al., 2010b), and both Vangl2 and Daam1 were reported to function in kidney tubulogenesis (Miller et al., 2011; Yates et al., 2010a). Whereas Wnt/Planar signaling was proposed to directly control endodermal cell ingression in Caenorhabditis elegans (Lee et al., 2006), mutations in the worm VANG-1 and FMI-1/Celsr homologs do not reveal gastrulation phenotypes (Sawa, 2012). Thus, it is currently unclear whether the proposed function of Vangl2 in apical constriction is conserved in invertebrates.

The involvement of Vangl2 in apical constriction differs from the known functions of PCP proteins in cell intercalations and in coordinating the orientation of a cell relative to its neighbors (Gray et al., 2009; Keller, 2002; Sokol, 2000; Wallingford, 2012). This multi-functionality of Vangl2 resembles the pleiotropic roles of actomyosin complexes, which function in different cellular compartments to mediate diverse cell behaviors (Heisenberg and Bellaiche, 2013; Tada and Kai, 2012). Although the number of
molecular players is limited, their specific interactions in a certain location within a cell must be crucial for the signaling outcome, such as the decision to migrate or to constrict the apical surface of the cell. Defining the molecular pathways controlling cell behavior will remain a major challenge for future studies of morphogenesis.

One possibility is that Wnt/PCP proteins directly modulate Myosin II activation and apical constriction through Rho-associated kinase (Marlow et al., 2002; Shindo and Wallingford, 2014; Winter et al., 2001) and one of the RhoGEFs, such as PDZ-RhoGEF (Nishimura et al., 2012) or GEF-H1, a RhoGEF that triggers ectopic apical constriction (Itoh et al., 2014). Consistent with this idea, we observed a decrease in MLC phosphorylation at the blastopore in Vangl2-depleted embryos. Although Vangl2 activity has been associated with the regulation of adherens junctions (Lindqvist et al., 2010), the direct link between Vangl2 and Rock or Myosin activation remains to be established. Alternatively, the Wnt/PCP pathway might modulate Myosin II activity in bottle cells through Rab11-mediated trafficking. The ability of overexpressed Rab11 to rescue blastopore defects in Vangl2-depleted embryos suggests that vesicular trafficking plays an essential role in PCP signaling during blastopore formation. The apical enrichment of PCP proteins in the blastopore cells might relate to the known functions of Rab11 and Myosin V in apical recycling in tissue culture cells (Bryant et al., 2010; Li et al., 2007; Roland et al., 2011). We note that apical protein localization has been previously associated with PCP signaling due to the apical distribution of Frz in Drosophila wing cells (Wu et al., 2004). Moreover, apical PCP protein distribution has been observed in the mouse node cells (Antic et al., 2010; Hashimoto et al., 2010; Mahaffey et al., 2013). Based on the effect of Vangl2 MO on Rab11 localization, we propose that PCP signaling plays a general role in apical constriction by targeting Rab11-positive recycling endosomes within the apical compartment. This hypothesis is supported by other studies implicating PCP components in the control of membrane dynamics and vesicular trafficking in Drosophila and vertebrates (Classen et al., 2005; Gray et al., 2009; Lee et al., 2003; Mahaffey et al., 2013). Although many molecules can serve as a cargo for Rab11 recycling vesicles, one possibility is that PCP proteins themselves are recycled to the cell surface to act at the site of apical constriction (Devenport et al., 2011). Our observations support a positive-feedback model in which PCP signaling and endocytic trafficking cooperatively function during apical constriction.

**MATERIALS AND METHODS**

**Plasmids, mRNA synthesis and morpholinos**

The plasmids encoding CFP-Vangl2/Stbm (Itoh et al., 2009), mouse HA-Vangl2 (Gao et al., 2011), mouse Shroom/ShrlM (also known as Shroom3) (Hildebrand and Soriano, 1999) and mouse FLAG-Shroom (Plageman et al., 2010; Chu et al., 2013) in pCS2 were as described previously. Zebrafish (Hildebrand and Soriano, 1999) and mouse FLAG-Shroom (Plageman et al., 2010; Chu et al., 2013) in pCS2 were as described previously. The sequence of the control MO was described previously. The sequence of the control MO was 5′-TACCCATCGTCGCGGCACTTCTGAC-3′. This study).

**Xenopus embryos, RNA injections and apical constriction**

In vitro fertilization, culture and staging of *Xenopus laevis* embryos were carried out as described previously (Dollar et al., 2005). For microinjections, four-cell embryos were transferred into 2% Ficoll in 0.3×MMR buffer, and 5 nl of mRNAs or MO solution was injected into one or more blastomeres. Amounts of injected mRNA or MO per embryo have been optimized in preliminary dose-response experiments (data not shown) and are indicated in figure legends. For ectopic apical constriction, four- to eight-cell embryos were injected animaly into one dorsal and one ventral animal blometrome. Embryonic defects were scored as the percentage of embryos with the representative phenotype, based on three to five independent experiments. Each experimental group contained 25-35 embryos.

**Immunohistochemistry and immunoblot analysis**

Anti-Vangl2 antibodies were generated by immunizing rabbits with the peptide corresponding to amino acids 56-70 of *Xenopus* Vangl2 and affinity-purified on a column with the covalently bound peptide. The 60 kDa Vangl2 band recognized by this antibody in *Xenopus* gastrula stage lysates was depleted in Vangl2 morphants (data not shown). The initial characterization of Vangl2 protein localization was performed using anti-Vangl2 antibodies provided by M. Montcouquiol (Université Bordeaux, France).

For cryosectioning, the embryos were embedded in solution containing 15% cold fish gelatin and 15% sucrose, sectioned at 10 μm and immunostained overnight, essentially as described previously (Dollar et al., 2005). Antibodies against the following antigens were used: Vangl2 (1:100, rabbit polyclonal, a gift from M. Montcouquiol; Montcouquiol et al., 2006); and this study, 1:100, rabbit polyclonal), Rab11 (1:100, Zymed, rabbit monoclonal; 1:100, Invitrogen, rabbit polyclonal; and 1:100, BD Biosciences, mouse monoclonal), GFP (1:200; B-2, Santa Cruz Biotechnology, mouse; or Invitrogen, rabbit), anti-phospho-Ser20 myosin light chain (anti-p-MLC, 1:300; Abcam, rabbit), β1-integrin (1:50; SC8, Dollar et al., 2005, mouse monoclonal). Secondary antibodies were against mouse or rabbit IgG conjugated to Alexa Fluor 488, Alexa Fluor 555 (1:100, Invitrogen) or Cy3 (1:100, Jackson ImmunoResearch). Cryosections were mounted for observation with the Vectashield mounting medium (Vector). For detection of Rab11 and pMLC on cryosections, embryos were devitellinized, fixed with 2% trichloroacetic acid (TCA) solution for 30 min at room temperature and washed with 0.3% Triton X-100 in PBS for 30 min (Nandadasa et al., 2009). Standard specificity controls were performed to confirm lack of crossreactivity and no staining without primary antibodies. Immunofluorescence images were captured using the Axio Imager fluorescence microscope (Zeiss) and the AxioVision imaging software (Zeiss). Results are shown as representatives from images from at least three independent experiments, each containing 10-20 embryos per group. For en face immunofluorescent imaging of the blastopore lip, stage 10+ embryos injected with control MO or Vangl2 MO with GFP RNA as a tracer were devitellinized, fixed for 1 h with MEMFA (0.1 M MOPS pH 7.4, 2 mM EGTA, 1 mM MgSO4 and 3.7% formaldehyde), washed with PBS and subjected to whole-mount immunostaining. Samples were blocked with 10% goat serum in PBS for 1 h at room temperature. For F-actin visualization, fixed embryos were co-stained with phalloidin conjugated to Alexa Fluor 568 (5 units/ml, Molecular Probes) and anti-GFP antibodies (1:200, B-2, Santa Cruz mouse monoclonal) overnight at 4°C in 10% goat serum in PBS. After washing, samples were incubated with anti-mouse-IgG secondary antibodies conjugated to Alexa Fluor 488 (Molecular Probes, 1:500) for 2 h in 10% goat serum in PBS. After washing, dorsal lip explants were dissected with a razor blade and mounted for observation in the Vectashield mounting medium (Vector).

Western blot analysis was carried out as described previously (Ossipova et al., 2009). Briefly, whole embryos or animal cap explants were lysed in a buffer containing 1% Triton X-100, 50 mM sodium chloride, 50 mM Tris-HCl pH 7.6, 1 mM EDTA, 0.6 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM sodium fluoride and 1 mM sodium orthovanadate. The lysates were subjected to SDS-polyacrylamide gel electrophoresis, and proteins were transferred to a PVDF membrane for immunodetection with rabbit polyclonal anti-Vangl2 antibody (Montcouquiol et al., 2006; and this study).
RT-PCR analysis
Total RNA was extracted from stage 10.5 embryos using an RNasey kit (Qiagen) according to the manufacturer’s instructions. cDNAs were made from DNase-treated RNA using the Superscript first-strand synthesis system (Invitrogen). The RT-PCR primers were as follows: Gsc forward, 5′-GAACACGTGGCAAGGAGAT-3′; Gsc reverse, 5′-GATCTTTT-TCTGCCCTCTCTCA-3′; Odc forward, 5′-CATCCTCCTACCCCTGGAC- TT-3′; and Odc reverse, 5′-CATCGGACAGCTTCTCTCTCA-3′; Gef3.2 (GenBank accession number NM_001096674.1) forward, 5′-AGTGGG-TTTCAAAATGGAT-3; TTT-C-3, and Gef3.2 reverse, 5′-CTTCCAACCTCCTC-TTGT-3′. These primers were as described previously, including Chordin (Hikasa and Sokol, 2004), FoxD3 (Ossipova et al., 2011) and Xbra (Agus et al., 2000), as were described previously.

The number of PCR cycles for each primer pair was determined empirically to maintain amplification in the linear range. One quarter of each PCR reaction was electrophoresed in 2% agarose gel, stained with ethidium bromide and photographed under ultraviolet light.

bromide and photographed under ultraviolet light.

PCR reaction was electrophoresed in 2% agarose gel, stained with ethidium bromide and photographed under ultraviolet light.

empirically to maintain amplification in the linear range. One quarter of each PCR reaction was electrophoresed in 2% agarose gel, stained with ethidium bromide and photographed under ultraviolet light.

Acknowledgements
We thank J. Goldenring, R. Habas, A. Hall, R. Lang, F. Marlow, P. Soriano, Y. Yang and M. Tada for plasmids, M. Montcouquiol and J. Ezan for anti-Vang2 antibodies, and B. Lake for the initial experiments with Rab11. We also thank members of the Sokol laboratory for discussions.

Competing interests
The authors declare no competing financial interests.

Author contributions
O.O. and S.Y.S. designed the experiments and wrote the manuscript. O.O. performed the experiments. I.C. designed and analyzed RT-PCR experiments, C.-W.C. generated and tested the zPk1 PL construct. All authors edited the manuscript.

Funding
This study was supported by National Institutes of Health grants [NS40972 and HD078874 to S.Y.S.]. Deposited in PMC for release after 12 months.

Supplementary material
Supplementary material available online at http://dev.biologists.org/lookup/suppl Đive1:111611/-DC1

References


Supplementary figure legends to Figures S1-S5.

**Fig. S1.** Immunodetection of endogenous Vangl2 protein.

(A, B) Basolateral localization (arrowhead) of Vangl2 (A) and β1-integrin (B) in embryonic ectoderm at stage 11. (C) Immunoblot of ectodermal cell lysates with anti-Vangl2 antibody reveals a 60 kDa band (arrowhead) corresponding to endogenous Vangl2, misexpressed *Xenopus* CFP-Vangl2 and mouse HA-VL2, as indicated.
(D-G) Vangl2 immunostaining on cross-sections of st. 10' and st. 10.5 embryos.

Dashed boxes in D and E correspond to the magnified images shown in D’ and E’.

Vangl2 is detected at the basolateral membrane and in cytoplasmic vesicles near the apical cortex, and appears more abundant in the superficial cell layer. Scale bar in A is 20 µm. Both apical (arrow) and basolateral staining is detected in the constricting blastopore cells of st. 10.5 embryo. D-G, Scale bar is 20 µm.
Fig. S2. Inhibition of blastopore lip formation in Vangl2-depleted embryos. (A-E)

Early embryos were injected with Vangl2 MO or control MO either dorsally or
ventrally as indicated. LacZ RNA (150 pg) has been coinjected a lineage tracer.

Vegetal views of stage 11 embryos are shown. E, Quantitation of the experiments
shown in A-D. (F, G) En face view of the dorsal lip in stage 10+ embryos. Vangl2 was
depleted by a unilateral injection of control or Vangl2 MO (30 ng each). F-actin
staining in control (F) and Vangl2-depleted (G) blastopore cells. Arrow points to
reduced apical F-actin in Vangl2-depleted cells (marked by GFP, green). Animal pole
is at the top. Scale bar in F (also applies to G) is 10 µm. Dashed line indicates the
midline. Arrowheads point to F-actin-enriched areas, which are reduced by Vangl2
depletion.
Fig. S3. Rab11 subcellular localization before gastrulation. (A-C) Cross-sections of late blastula embryos (n=10) stained for endogenous Rab11. A, Representative embryo at stage 9.5 is shown, B, Dorsal marginal zone area reveals enriched apical (asterisk) and apical junctional (arrowhead) staining. C, Rab11 is mostly at the junctions (arrowheads) in the ventral marginal zone. Scale bar is 20 µm in all panels.
Fig. S4. Rescue of dorsal blastopore in Vangl2-depleted embryos by Rab11 RNA.

(A, B) Embryos were injected with 30 ng of Vangl2 MO and 1 ng of Rab11Q70L RNA as indicated. (C) Uninjected control embryo. Vegetal views reveal the comparative morphology of the dorsal lip (arrow) for representative embryos from data in Fig. 6I.
Fig. S5. Vangl2 is dissociated from the cell membrane in Rab11-depleted embryos.

Embryos were co-injected with CO MO or Vangl2 MO (30 ng each) and 100 pg of GFP RNA as lineage tracer (not shown). A, B, Morphology of control (A) and Rab11-depleted (B) embryos at st. 11, vegetal views are shown. C, D, Rab11 depletion interferes with the membrane localization of Vangl2, including the apical surface of the constricting blastopore cells (arrow). E, F, Basolateral membrane localization of Vangl2 is inhibited in ectoderm cells depleted of Rab11. Scale bar in E, also refers to C, D and F, is 20 µm.